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PATHOGENESIS OF HEPATOCELLULAR CARCINOMA DEVELOPMENT IN NON ALCOHOLIC FATTY LIVER DISEASE

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is being recognized as an increasingly important contributor to the burden of hepatocellular carcinoma (HCC) worldwide. It is often accompanied by obesity and diabetes mellitus and is believed to be the hepatic representation of the metabolic syndrome. HCC development in NAFLD is multifactorial and complex. It is dependent on not only the well-described mechanisms noted in chronic liver injury, but also on the molecular derangements associated with obesity and dysmetabolism. These include adipocyte remodeling, adipokine secretion, lipotoxicity and insulin resistance. Recent advances focus on the importance of the gut-liver axis in accelerating the process of oncogenesis in NAFLD. The farnesoid X nuclear receptor (FXR) has been demonstrated to have important metabolic effects and its pharmacological activation by obeticholic acid has been recently reported to produce histological improvement in NASH. It is hoped that delineating the mechanisms of hepatic fibrosis and oncogenesis in NASH will lead to enhanced strategies for cancer prevention, surveillance and therapy in this population.

Keywords

Hepatocellular carcinoma; non-alcoholic fatty liver disease; pathogenesis

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Conflict of Interest

Kirti Shetty, Jian Chen, Ji-hyun Shin, Wilma Jogunoori and Lopa Mishra declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

1. INTRODUCTION

Definitions and diagnosis

Non-alcoholic fatty liver disease (NAFLD) is a clinico-pathological entity that encompasses a continuum of findings. It ranges from the uncomplicated accumulation of triglycerides within hepatocytes (steatosis/nonalcoholic fatty liver (NAFL)) to a histologically distinct entity characterized by hepatic inflammation and hepatocyte damage denoted as non-alcoholic steatohepatitis (NASH). Before making a diagnosis of NAFLD, other causes of hepatic steatosis require exclusion: these include hepatitis C virus (HCV) infection (of which genotype 3 disease is associated with hepatic steatosis), alcohol overuse, use of steatogenic medications, or hereditary disorders [1]. The minimal lesions required for a diagnosis of NASH include ≥5% macrosteatosis, inflammation, and ballooning; the latter occurs primarily in acinar zone 3 [2]. No reliable biomarkers exist to distinguish NAFL from NASH, a distinction that is clinically relevant, since only 3% of NAFL patients progress to clinically significant consequences of liver disease in the form of cirrhosis and hepatocellular carcinoma (HCC), compared to 25% of those with NASH [3].

2. EPIDEMIOLOGY

2.1 Prevalence of NAFLD

NAFLD is now believed to be the most prevalent cause of chronic liver disease in the West and is becoming increasingly common in Asia, where changing economies, diets and lifestyles have led to a rapid increase in the rates of obesity and diabetes. NAFLD prevalence in a particular population varies widely according to the method of assessment as well as the population studied. Liver biopsy, while the gold standard, cannot be utilized in population based studies. Data from the US National Health and Nutrition Examination Survey 1988 – 1994 (NHANES) utilized ultrasonographic evidence of hepatic steatosis to define NAFLD and found a prevalence of 19% [4]. A prospective study utilizing both ultrasound and liver biopsy found overall NAFLD prevalence rate of 46% with NASH confirmed in 12.2% of the cohort [5]. Studies from other parts of the world report prevalence rates of 13 – 30% [6, 7]. Obesity, diabetes mellitus and the metabolic syndrome, are common associations in most series, with the noticeable exception of studies from Asia where NAFLD has been reported in non-obese populations [8, 9].

NASH can lead to cirrhosis in about a quarter of those affected, with approximately 10–15% of those with cirrhosis developing hepatocellular carcinoma (HCC) [3]. It may therefore be anticipated that the steady increase in overall rates of the metabolic syndrome and its components will potentially place a large number of individuals at risk for NASH and its complications, including HCC.

2.2 Relationship between NAFLD and HCC

Multiple studies have implicated NAFLD as a risk factor for HCC development. A recent meta-analysis summarized epidemiologic data to conclude that the increasing burden of HCC could be linked to the increasing prevalence of NAFLD [10]. It is estimated that between 4 – 22% of HCC in the West is attributable to NAFLD; in Asia where viral

hepatitis is endemic, NAFLD-associated HCC accounts for 1–2% of cases. However, these estimates do not take into account the HCC that occurs against a background of “cryptogenic” cirrhosis (CC). Multiple studies confirm that risk factors for NASH, including diabetes, obesity and dyslipidemia, were all noted to be significantly increased in those with CC [11–13]. In these series, CC was the underlying disease in between 6.9% [11] to 29% of HCC cases [14]. HCC associated with both CC and NAFLD correlate with older age, and less aggressive tumor behavior as compared to HCC related to HCV [15].

Cirrhosis is believed to be a crucial intermediary during the process of hepatocarcinogenesis in NASH. Epidemiologic data has found that the risk of HCC within NAFLD cohorts varies according to the proportion of cases with cirrhosis. Cohorts with few cases of cirrhosis had low cumulative HCC mortality rates of 0%–3% while those with cirrhosis had a consistently higher risk (cumulative incidence ranging from 2.4% to 12.8% over 3 years) [10]. Genome wide association studies (GWAS) have identified a patatin-like phospholipase domain containing 3 (PNPLA3) gene variant as a marker of advanced fibrosis and HCC in NASH [16], corroborated by a recent meta-analysis [17]. The mechanisms behind HCC susceptibility conferred by this genetic variant are yet to be defined.

2.3 Relationship Between Obesity, Metabolic Syndrome and HCC

Obesity is emerging as the leading public health challenge of the developed world. According to 2008 World Health Organization (WHO) estimates, 35% of adults were overweight (body mass index (BMI) ≥ 25 kg/m²) and 12% were obese (BMI ≥ 30 kg/m²), a prevalence of 1.4 billion overweight adults, of whom 500 million were obese. The WHO Regions of the Americas had the highest prevalence rates of overweight and obese adults. [18].

Obesity is a central element of the metabolic syndrome which is defined by the U.S. National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) as the presence of at least three of the following conditions: elevated waist circumference/central obesity, dyslipidemia (elevated triglycerides, lowered high-density lipoprotein), hypertension, and impaired fasting glucose [19]. NAFLD is believed to be the hepatic expression of the metabolic syndrome, and is particularly prevalent in those who are obese, diabetic or both. Population based cohorts such as the European Prospective Investigation into Cancer and Nutrition (EPIC) suggest that obesity may play a role in 16% of HCC cases [20]. Other epidemiological studies have confirmed the association between BMI and HCC with each 5kg/m² BMI increase conferring an average 24% increased risk of liver cancer [21–23]. In a recent systematic review, diabetic subjects were found to have a doubled risk of HCC [24]. An analysis of the Surveillance, Epidemiology, and End Results (SEER)-Medicare database demonstrated that individual components of the metabolic syndrome were each significantly associated with HCC development ($P < 0.0001$) and in combination significantly increased the odds of HCC (37.1% versus 17.1%, $P < 0.0001$) [25].

2.4 HCC Development in NAFLD without Cirrhosis

Most NAFLD associated HCC occurs against a background of cirrhosis, but accumulating evidence – both epidemiological and pathological – suggests that HCC in NAFLD may

occur without cirrhosis. A recent scrutiny of health utilization claims from the US identified NAFLD as the most common underlying risk factor for HCC, with less than half (46%) of NAFLD associated HCC documented to have underlying [26].

A large pathological series of non-cirrhotic HCC associated with NAFLD was described from Japan, wherein 87 HCC cases were found against a background of steatohepatitis without cirrhosis [27]. A French series of resected HCC found that those cancers occurring against a non-cirrhotic background were more likely to be associated with unclear etiology of liver disease [28]. An updated HCC cohort from the same group reported that patients without an identifiable etiology of liver disease, but with underlying metabolic syndrome, were more likely to have minimal or no fibrosis as compared to individuals with a defined etiological cause for liver disease. [29]. These observations raise the possibility that HCC in NAFLD may arise in the absence of cirrhosis and if confirmed, have profound implications for HCC surveillance in NAFLD.

3. MECHANISMS OF HEPATOCARCINOGENESIS IN NAFLD

3.1 Overview

HCC development in chronic liver disease is described as a step by step process, whereby chronic inflammation, tissue damage, regeneration, remodeling and uncontrolled proliferation lead to carcinogenesis. The mechanisms behind HCC development in cirrhosis have been reviewed exhaustively in recent publications [30] and implicate multiple oncogenic pathways, with the identification of aberrant signaling and major genomic defects. Chronic inflammation is believed to be a key intermediary in the development of HCC. The liver exists within a unique pro-inflammatory microenvironment – it contains several immunologically active cell types such as the Kupffer cells, liver dendritic cells, and T cells [31, 32]. Parenchymal hepatocytes and nonparenchymal cells such as the hepatic stellate cells (HSC) and liver sinusoidal endothelial cells (LSECs) function as antigen presenting cells. When a triggering agent presents itself, it activates pattern recognition receptors such as the membrane-bound Toll-like receptors (TLRs) and C-type lectins which in turn induce a cascade of signals leading to the production of proinflammatory cytokines. [32]

Clues to the link between inflammation and HCC may be inferred from the inflammatory mouse model of inflammation (Mdr2-knockout strain) wherein the tumor necrosis factor (TNF)- nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) axis was found to have a tumorigenic effect on the liver, with interference of this circuitry inhibiting tumor progression [33]. Studies of human HCC also confirm that NFκB activation is an important initiating event in hepatocarcinogenesis [34, 35]. Signal transducer and activator of transcription 3 (STAT3) is reported to be a crucial link between inflammation and carcinogenesis. In a mouse model of obesity induced liver disease, the proinflammatory cytokines (TNF and interleukin 6 (IL6)) lead to STAT3 activation which in turn promotes malignant transformation [36]. In human HCC STAT3 activation has been found to correlate with tumor aggressiveness [37].

Hepatocarcinogenesis is a molecularly heterogeneous process. Initial epigenetic changes lead to aberrations of DNA methylation on CpG groups. [30] Subsequent genomic changes include point mutations and chromosomal gains. Recent landmark studies utilizing next generation sequencing have highlighted the following key findings with regard to the HCC exome: the Wnt/ β catenin pathway is commonly implicated, and accounts for 48% of all mutations in HCC: either β catenin activating mutations(32%) or AXIN1/APC inactivating mutations (16%). Genes involved in p53/cell cycle check point control are also involved, with inactivation of the TP53 gene / encoding interferon regulatory factor 2 (IRF2) genes, and subsequent downstream interaction with mdm2, an inhibitor of p53. Additional gene alterations appear in the chromatin remodelers ARID1A and ARID2, as well as the p13K and K Ras signaling pathway and those involved in oxidative stress [38–40]. This complexity results in resistance to therapeutic interventions and the poor prognosis associated with HCC.

3.2 Mechanisms of Hepatocarcinogenesis in Metabolic Syndrome

Cirrhosis accounts for the majority of cases of NAFLD associated HCC, but as noted above, HCC in NAFLD may occur in the absence of cirrhosis. A possible explanation for this phenomenon is that obesity and dysmetabolism serve as drivers of oncogenesis in the setting of abnormal hepatic morphology, and that hepatic steatosis may provide the appropriate microenvironment for the development of cancer. Potential mediators of obesity related HCC include lipotoxicity, dysregulation of pro-inflammatory / anti-inflammatory cytokines(specifically, reduced adiponectin/increased leptin) and hyperinsulinemia leading to stimulation of the insulin like growth factor (IGF1). The relative contributions of each of these is discussed in the following sections and illustrated in figure 1.

3.2.1 Adipocyte remodeling and cytokine secretion—Obesity is characterized by the expansion and remodeling of adipose tissue. This results in a chronic inflammatory state, characterized by an altered pattern of cytokine secretion by adipocytes. These adipose-derived cytokines or adipokines include leptin and adiponectin. Other implicated cytokines are tumor necrosis factor alpha (TNF α), transforming growth factor beta (TGF β), interleukin 6 (IL-6) and interleukin 1 beta (IL 1 β). TNF α induces several pro-oncogenic pathways, including NF- κ B; of note, obesity has been demonstrated to increase TNF α and IL-6 levels in malignant and non malignant tissue. Elevated IL-6 and TNF α levels regulate gene expression through STAT3, recognized as a driver oncogene [41]. In an experimental model of dietary obesity, TNF and IL-6 derived from adipose tissue were implicated in the promotion of diethylnitrosamine-induced HCC possibly by the upregulation of both extracellular signal-regulated kinase (ERK) and STAT3 pathways [42].

Leptin has been demonstrated to have potent pro inflammatory and pro-fibrogenic actions. Its growth promoting effects are mediated by the by Janus kinase (JAK), STAT3, phosphoinositotide 3-kinase (P13K)/protein kinase B (Akt) and ERK signaling pathways [43]. Adiponectin, which is decreased in obesity, suppresses tumor angiogenesis and appears to inhibit HCC growth and metastasis [44]. Adiponectin exerts its effects by activation of 5'adenosine monophosphate activated protein kinase (AMPK) which acts as a tumor suppressor. It also increases cell apoptosis by regulating the mammalian target of rapamycin

(mTOR) pathway and c-Jun N-terminal kinase (JNK)/caspase 3 pathways. This counterbalance between the opposing effects of leptin and adiponectin is postulated to have a crucial role in oncogenesis associated with hepatic steatosis.

3.2.2 Role of Lipotoxicity—Sustained nutrient excess leads to abnormal fat deposition in non-adipose tissues, giving rise to cellular dysfunction referred to as lipotoxicity. The liver is particularly at risk for ectopic lipid accumulation due to several factors, notably the increased supply of dietary lipids delivered via the portal vein in obesity. The expression of phosphatase and tensin homologue (PTEN) is inhibited by the accumulation of unsaturated fatty acids in hepatocytes. PTEN acts as a tumor suppressor and also regulates phosphoinositide 3-kinase (PI3K) signaling. PTEN has been noted to be either mutated or deleted in HCC [45]. Lipotoxicity also mediates several pro-oncogenic changes such as mitochondrial dysfunction, increased fatty acid oxidation in peroxisomes / microsomes and the generation of free reactive oxygen species [46].

3.2.3 Insulin resistance—Obesity leads to the development of both hepatic and systemic insulin resistance, and is worsened by hepatic lipid accumulation. Prolonged hyperinsulinemia stimulates the production of IGF binding protein, increasing the bioavailability of IGF1 and IGF2 [47]. High insulin levels and IGF levels may promote cancer development by activating oncogenic pathways involving PI3K/Akt, mitogen-activated protein kinase (MAPK) and vascular endothelial growth factor (VEGF) [48].

Additionally, sterol regulatory element binding proteins (SREBPs) which are instrumental in the regulation of lipogenesis within the liver are activated by hepatic lipid accumulation. In HCC, SREBP1 has been shown to markedly induce lipogenesis, and correlates with a poor prognosis [49].

4. CONTRIBUTORY PATHWAYS TO HEPATOCARCINOGENESIS IN NAFLD

4.1 Role of Gut Microbiota

It is hypothesized that gut microflora may promote hepatic steatosis and NASH development by the following mechanisms: regulation of gut permeability, maintenance of inflammation, modification of dietary choline metabolism and dysregulation of bile acid metabolism [50].

The gut microbiota participates in the maintenance of host immunity. Specific receptors, including TLRs and nucleotide-binding oligomerization domain receptors (NOD)-like receptors act as immune sensors of microbial molecules and endogenous products designated as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) respectively [51]. A crucial mediator of the gut microbiome is the inflammasome, which comprises leucine-rich-repeat containing proteins/nucleotide binding domains (NLRPs) and acts as a sensor of PAMPs and DAMPs [52]. Henao-Mejia et al have recently shown that inflammasome deficiencies in mice were associated with exacerbated hepatic steatosis, and co-housing transferred the phenotype to wild-type mice, hence supporting the role of the inflammasome in NASH progression [53]. NAFLD has been shown to be linked to small intestinal bacterial overgrowth (SIBO) and increased

intestinal permeability [54]. Additionally, gram negative bacteria which produce lipopolysaccharide (LPS) are selectively enriched in the intestines of obese humans and rodents [54]. LPS is a potent PAMP, activating TLR-4 and triggering an inflammatory cascade involving pro-oncogenic pathways [55] (Figure 1). These events provide putative evidence of the link between gut dysbiosis and hepatocarcinogenesis in NAFLD.

4.2 Senescence Associated Secretory Phenotype

The process of cellular senescence within HSCs is recognized as a homeostatic mechanism within the liver that regulates proliferation and suppresses oncogenesis [55]. However, recent data suggests that senescent cells may generate multiple cytokines and growth factors (termed the senescence-associated secretory phenotype or SASP), which may contribute to tumorigenesis [55]. Yoshimoto et al have demonstrated in an obese mouse model that HSC senescence was increased by administration of the liver carcinogen dimethylbenz[a]anthracene [56]. Conversely, depletion of senescent HSCs caused decreased tumorigenesis. [56].

4.3 Bile Acids

Diets high in saturated fats have been demonstrated to alter the composition of bile acids and may alter rates of transit for gut microbes, leading to dysbiosis. The gut microbiota, in turn, affects the metabolism of bile acids, specifically by stimulation of the Farnesoid X receptor (FXR) which plays an important role in lipid metabolism within hepatocytes. In mice, FXR loss has been shown to have profound effects in the form of higher plasma lipids, steatohepatitis and malignant transformation of hepatocytes [57–59].

Alterations in gut microbiota increase levels of deoxycholic acid (DCA), a bile acid linked to DNA damage. In the obese mouse model of HCC noted above [56], targeted elimination of gut bacteria by vancomycin or reduction of DCA levels suppressed HCC development. Intriguingly, lowering DCA concentration was associated with a reduction in senescent HSC suggesting a mechanistic link between altered gut microbiota, bile acids and stellate cell senescence-associated promotion of HCC in obesity.

4.4 Role of Vitamin D (VD) and the Vitamin D Receptor (VDR)

VD is increasingly being recognized as a modulator of immunity, cell differentiation and proliferation and its deficiency is linked to multiple disease states, including NAFLD [60, 61]. Epidemiological data linking VD deficiency (VDD) and HCC development is contradictory. A case-control study from China examining liver cancer in liver disease appeared to negate an association between the risk of liver cancer and circulating levels of VD [62]. In contrast, a recent sub-analysis of the EPIC cohort reports that VDD bestows an increased HCC risk of 1.82 compared to reference subjects [63].

It has been recently demonstrated that upon stimulation by vitamin D receptor (VDR) ligands, nuclear VDR binds to the pro-fibrogenic genes it co-regulates with SMAD3, reducing SMAD3 occupancy at these sites and inhibiting fibrosis [64]. *Vdr* knockout mice have been reported to spontaneously develop hepatic fibrosis.

The effect of VDD in animal models has been investigated by Roth et al, wherein VDD predisposed rats fed a high fat diet to develop hepatic steatosis and increased lobular inflammation [65]. Adipokines such as leptin, resistin, TNF- α and IL-6 levels were increased in VD deficient rats and adiponectin levels were decreased suggesting that VDD up-regulates hepatic inflammatory and oxidative stress genes [65]. VDD also led to increased expression of TLR-2, 5 and 9, which may mediate increased endotoxin exposure to the liver [65]. VD analogs have been shown to have growth inhibitory effects on HCC cell lines, and chemopreventive effects in a mouse model of HCC [66]. Further studies are required to evaluate the beneficial properties, if any, of VD supplementation on hepatic histology, inflammation and carcinogenesis in NAFLD, but experimental evidence suggests that VD and its analogs may have an anti-steatogenic and growth inhibitory effect on hepatocytes and HCC. Figure 1 illustrates this interplay and possible therapeutic targets.

FUTURE DIRECTIONS AND THERAPEUTIC TARGETS

One of the many challenges in dealing with NAFLD is the paucity of effective treatments. A potential new therapy is obeticholic acid (OCA; INT-747, 6 α -ethyl-chenodeoxycholic acid). OCA is a semisynthetic derivative of the primary human bile acid chenodeoxycholic acid and the natural agonist of the farnesoid X receptor. [67] A phase 2 trial of OCA in humans demonstrated increased insulin sensitivity, and reduced markers of liver inflammation and fibrosis in patients with DM type 2 and NAFLD. [67]. A recently concluded multicenter randomized controlled trial (Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment (FLINT) trial) randomized patients with biopsy proven NASH to 72 weeks of obeticholic acid versus placebo [68]. Forty five percent of those in the OCA group had improved liver histology compared with 21% of patients in the placebo group (relative risk 1.9, 95% CI 1.3–2.8; $p=0.0002$). [68]. These results await further confirmation. Other therapeutic approaches target SIBO that appears to underlie NAFLD. In a recent study, Dapito et al have proposed that HCC could be prevented by gut sterilization in individuals with chronic liver disease. [69]. In another novel attempt to modulate gut microbiota, Vrieze et al concluded a randomized controlled trial in patients with metabolic syndrome wherein infusion of lean-donor feces was demonstrated to improve hepatic and peripheral insulin resistance [70].

CONCLUSIONS

The increasing incidence of obesity and metabolic syndrome in much of the developed world has led to a rising global burden of HCC, which is likely to accumulate further in the coming decades. Of concern, HCC may occur in NAFLD without the intermediary process of severe fibrosis or cirrhosis, hence complicating efforts at surveillance and early detection. Fibrosis, cirrhosis and chronic inflammation are well-recognized precursors to the development of HCC in NAFLD. It is also now evident that obesity and its sequelae such as insulin resistance, adipose remodeling, and alterations in the gut microbiota, are important intermediaries in the initiation and propagation of oncogenesis in NASH. It is therefore crucial to understand the molecular pathways that lead to carcinogenesis in obesity, and to develop public health strategies that address the burgeoning epidemics of diabetes, obesity and NAFLD.

ABBREVIATIONS

NAFL(D)	Non-alcoholic fatty liver (disease)
NASH	non-alcoholic steatohepatitis
HCV	hepatitis C virus
NHANES	National Health and Nutrition Examination Survey
HCC	hepatocellular carcinoma
CC	cryptogenic cirrhosis
GWAS	Genome wide association studies
PNPLA3	patanin-like phospholipase domain containing 3
WHO	World Health Organization
BMI	body mass index
NCEP-ATP III	National Cholesterol Education Program Adult Treatment Panel III
EPIC	European Prospective Investigation into Cancer and Nutrition
SEER	Surveillance, Epidemiology, and End Results
HSC	hepatic stellate cells
LSECs	liver sinusoidal endothelial cells
TLRs	Toll-like receptors
TNF	tumor necrosis factor
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
STAT3	Signal transducer and activator of transcription 3
IGF	insulin like growth factor
TGF β	transforming growth factor beta
IL-6	interleukin 6
IL1 beta	interleukin 1 β
ERK	extracellular signal-regulated kinase
JAK	Janus kinase
P13K	phosphoinositotide 3-kinase
Akt	protein kinase B
AMPK	5'adenosine monophosphate activated protein kinase
mTOR	mammalian target of rapamycin
JNK	c-Jun N-terminal kinase
PTEN	phosphatase and tensin homologue

MAPK	mitogen-activated protein kinase
VEGF	vascular endothelial growth factor
SREBPs	sterol regulatory element binding proteins
NOD	nucleotide-binding oligomerization domain receptors
PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
NLRPs	nucleotide binding domains
SIBO	small intestinal bacterial overgrowth
LPS	lipopolysaccharide
SASP	senescence-associated secretory phenotype
SMAD	small 'mothers against' decapentaplegic'
FXR	Farnesoid X receptor
DCA	deoxycholic acid
VD	Vitamin D
VDR	Vitamin D Receptor
VDD	VD deficiency
OCA	obeticholic acid
SIBO	small intestinal bowel overgrowth
(F)FFA	(Free) Fatty Acids
TLR4	Toll-Like Receptor4
TNF-α	tumor necrosis factor alpha
P13K	phosphoinositide 3-kinase
Gro-α	Growth-related oncogene protein-alpha
CXCL9	CXC ligand 9

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FIGURE 1.
The Gut-Liver Axis and HCC Pathogenesis in NAFLD